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Use of secondary ion mass spectrometry to image ^{44}calcium uptake in the cell walls of apple fruit

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Summary. Calcium, an important agent in regulating cell wall autolysis during fruit ripening, interacts with pectic acid polymers to form cross-bridges that influence cell separation. In the present study, secondary ion mass spectrometry (SIMS) was used to determine whether the cell walls of apple fruit were able to take up exogenously applied ^{44}Ca, which was infiltrated into mature fruit. SIMS, which has the ability to discriminate between isotopes, allowed localization of the exogenously applied ^{44}Ca and the native ^{40}Ca. The results indicated that the total amount of calcium present in the cell walls was enriched with ^{44}Ca and that heterogeneity of ^{44}Ca distribution occurred in the pericarp. Isotope ratio images showed microdomains in the cell wall, particularly in the middle lamella intersects that oppose the intercellular spaces. These domains may be the key areas that control cell separation. These data suggest that exogenously applied calcium may influence cell wall autolysis.

Keywords: Autolysis; Secondary ion mass spectrometry; Ripening; ^{44}Calcium; Cell separation; Intercellular space.

Abbreviations: SIMS secondary ion mass spectrometry.

Introduction

In plant cells, calcium has two distinct functions. Cytosolic free calcium operates as a messenger in signal transduction and as an intercellular regulator (Hepler and Wayne 1985, Gilroy et al. 1993, Poovaiah and Reddy 1993). This role is quite distinct from that of apoplastic calcium, which is involved in cross-bridging pectin and may serve an important structural role in the plant cell wall. The potential interaction between pectic substances and calcium ions has been studied extensively (Rees 1977; Gidley et al. 1980; Morris et al. 1982; Powell et al. 1982, Jarvis 1984, Thibault and Rinaudo 1985, 1986; Braudo et al. 1992). These investigations have established that this process includes the coulombic interactions with a polyanionic chain and coordinative binding. The evidence also suggests that ionically bound calcium is involved in regulating plant senescence and fruit ripening (Ferguson 1984, Brady 1992). As a result, increasing the calcium content in many climacteric fruit tissues retards the rate of fruit softening as well as other changes that are related to the ripening process (Poovaiah et al. 1988).

Unfortunately, a complete understanding of the role of calcium in regulating cell wall autolysis is lacking. Cell separation, which is one of the major cellular events involved in softening of the fruit, is no longer thought to be an uncontrolled process but rather a carefully regulated molecular event (Knox 1992). Supporting evidence for the role of acidic pectin and calcium in cell adhesion has been provided by several investigators (Fry 1988; Van Buren 1991; Knox 1992; Roy et al. 1992, 1994a; Jauneau et al. 1992a; Liners and Van Cutsem 1992). However, further clarification is required to explain how calcium is distributed in the cell wall of fruit and how exogenously applied calcium can affect cell wall degradation. A recent study used cationic colloidal gold for microscopic examinations to determine that the frequency and distribution of anionic binding sites in the walls of parenchyma cells of the apple were influenced by

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exogenous calcium (Roy et al. 1994b). The results indicated that infiltrated calcium decreased the number of anionic sites in the cell wall. The authors concluded that calcium could reduce fruit softening by strengthening the cell wall and limiting cell separation.

Another approach for investigating the role of calcium has been to localize it in situ by using different techniques. In situ precipitation of calcium with potassium antimonate has been reviewed by Wick and Hepler (1982). Fluorescent dyes and the luminescent photoprotein aequorin were used for imaging cytosolic free calcium in plant and fungal cells (Callaham and Hepler 1991; Read et al. 1992, 1993; Hepler and Callaham 1993; Gilroy et al. 1993). Bound calcium, which occurs in the cell walls at concentrations much higher than those found in the cytoplasm, has been visualized in situ using electron dispersive X-ray microanalysis (Burns and Pressey 1987) and electron energy-loss spectroscopy/electron spectroscopic imaging (Busch et al. 1993).

Recently, secondary ion mass spectrometry (SIMS) has been used for elemental characterization of biological tissues and cells (Burns 1982, Theillier et al. 1991, Linton and Goldsmith 1992, Chandra et al. 1994). SIMS was used to visualize calcium in the amyloplasts of the root cap cells (Chandra et al. 1982), in the nucleus during interphase and mitosis (Chandra et al. 1984), and in the cell walls of differentiating tissues (Jauneau et al. 1992a, b; Roy et al. 1994a). Other diffusible ions, such as sodium, nitrogen, and potassium, have also been visualized with SIMS (Lazof et al. 1992; Jauneau et al. 1992b, 1994; Ripoll et al. 1993). Mineral cations have been determined in cell walls of flax by using the depth profiling feature of SIMS (Ripoll et al. 1992). Because SIMS has the unique capability to discriminate isotopes based on their mass to charge ratio, isotopic gradients can be imaged in relation to tissue morphology (Chandra et al. 1990). Although this application has been extensively used for medical and pharmacological studies (Hindie et al. 1992, Linton and Goldsmith 1992), nutrient tracers have only been used in a few plant nutrition studies (Schaumann et al. 1986, Lazof et al. 1992, Grignon et al. 1992).

In the present study, we have used ion microscopy and the ^{44}Ca isotope in an attempt (*i*) to understand the process of calcium uptake by cell walls of mature fruit and (*ii*) to search for microdomains of calcium that may affect the etiology of cell wall separation.

Materials and methods

Plant materials and calcium infiltration

"Golden Delicious" apples (*Malus domestica* Borkh.) were harvested from a commercial orchard in Pennsylvania. Whole, undamaged fruits were infiltrated under 103 kPa of pressure for 3 min by submerging them in either distilled H_2O or 4% $^{40}CaCl_2$. After treatment, the fruit were allowed to drain for 2 h before storage at 0 °C. Because of the excessive cost of ^{44}Ca, other fruit were vacuum infiltrated for 3 min with 20 µl of a 2% $^{44}CaCl_2$ (98.89% ^{44}Ca enrichment; Oak Ridge National Laboratory, Oak Ridge, TN) by placing this solution in a small circular well (diameter ca. 0.8 cm) that had been formed with paraffin on the surface of the fruit. These small areas of infiltration were carefully delineated for further sampling. To determine the effectiveness of this technique and to obtain an indication of the extent and distribution of the perfused calcium, a solution of 0.5% fast green in water was deposited in the paraffin well of other control fruit and vacuum infiltrated.

Tissue preparation

After 1 month of storage, 2–3 mm^3 samples of pericarp comprising cuticle, epidermis, hypodermis and outer cortex were cut from the apple fruits and prepared for examination. Two methods of tissue preparation were used: (1) samples were vacuum infiltrated with a conventional chemical fixative, consisting of 2.5% glutaraldehyde in 0.1 M cacodylate buffer. This procedure was followed by post-fixation in 1% osmium tetroxide as described by Roland and Vian (1991); (2) samples were rapidly plunge-frozen in Freon 22 slush (ca. –160 °C) cooled with liquid nitrogen. These specimens were then transferred for freeze-substitution to 15 ml cryogenic vials filled with a solution of 2% (w/v) osmium tetroxide in acetone at –90 °C. The vials were placed in wells that had been drilled in an aluminum block. The aluminum block was put into a pre-cooled brass chamber that was then placed into an insulated encasement that had been pre-cooled with liquid nitrogen. The insulated encasement was filled with dry ice to maintain a temperature of –80 °C. The temperature of the sample block was monitored with a thermocouple that was placed in a vial in the center well of the aluminum block. Substitution with the osmium solution was allowed to proceed for 3 days. Subsequently the solution was slowly warmed (2 h at –60 °C, 2 h at –18°C, 2 h at –4 °C, and 2 h at room temperature), the substitution medium was discarded and the samples were rinsed in acetone.

Embedding and sectioning

To ensure proper resin infiltration of the cell walls, samples that had been conventionally prepared and those that had been freeze-fixed and freeze-substituted were infiltrated with Spurr's low viscosity resin according to the procedure described by Roland and Vian (1991). All embedded samples were polymerized at 60 °C for 3 days. The blocks were then sectioned and 1–2 µm thick sections were collected and mounted on polished carbon substrates for ion microscopic imaging. The sections were stabilized by sputter coating with a platinum film in an Oxford CT-1500 HF Cryotrans System. Mounting alternate sections on a glass slide and staining them with toluidine blue allowed direct light microscopic observations of adjacent tissues (Roland and Vian 1991).

Secondary ion mass spectrometry (SIMS)

Image analysis was performed on the Cameca IMS 4F using a positively charged oxygen primary beam. Details of this technique have

been reported by Burns (1982) and Thellier et al. (1991). Briefly, the technique consists of bombarding the surface of the specimen with a beam of primary ions (O_2^+ was used in this study). As a result of this bombardment, the atoms from the most superficial layers of the specimen are sputtered as neutral or charged particles (positive or negative). The charged particles or secondary ions are collected and separated by their mass/charge in a mass spectrometer (Burns 1982). Two configurations are possible for imaging the sputtered secondary ions. With ion microscopy in which optics are used, the ions retain the spatial configuration that they originally held on the sample surface. The secondary ions with the selected mass are focused by a system of projection lenses onto a microchannel fluorescent screen. The images obtained by this procedure are stigmatic images and were digitized using a scientific grade slow scan charged-coupled-device (CCD) camera. By using this procedure, the distribution of several isotopes can be determined from the same specimen because the analysis is processed by sputtering the sample surface in the z direction. In our study, all analyses were performed using a 1 μA positive oxygen primary beam at 12.5 keV with detection of positive secondary ions. The SIMS IMS 4F instrument can also be used in a microprobe mode using a small diameter primary ion beam, having a spot size of 0.25–0.5 μm. An ion image is formed by scanning the section with the beam. The intensity of the signal, which is measured by the electron multiplier, is used to modulate the intensity of the image on the CRT. To minimize variations due to the sputtering, different sequences of image recording were used: images of ^{40}Ca and ^{44}Ca were alternatively recorded starting either with ^{40}Ca or ^{44}Ca. The number of ^{40}Ca and ^{44}Ca images depended on the thickness of a particular section. The microprobe mode was employed to obtain an image ratio of $^{40}Ca/^{44}Ca$. High-resolution mass scans were performed to check for mass interference components in the secondary ion signals.

Results

Landmarks for topographical orientation

The fast green solution that was deposited in the paraffin well and vacuum infiltrated, stained a core of pericarp tissues 0.9 to 1 cm wide and 0.6 to 0.8 cm deep (data not shown). The histological features of the apple pericarp are illustrated in a transverse section (Fig. 1). The pericarp consists of three zones: (I) the epidermis which is covered by a thick cuticle, (II) three to four layers of small parenchyma cells that are tightly joined, and (III) large parenchyma cells with thin walls. The contiguous cell walls of the parenchyma vacuolated cells frequently separate along portions of their middle lamella and thereby form the numerous intercellular spaces that characterize this tissue. Sections from samples that were freeze-fixed and freeze-substituted exhibited the same gross morphological features indicating that these processing procedures did not significantly differ (data not shown). Together, the three distinct zones described above occur in the outer 250 μm layer of the pericarp. All three zones were included in the 0.6 cm depth

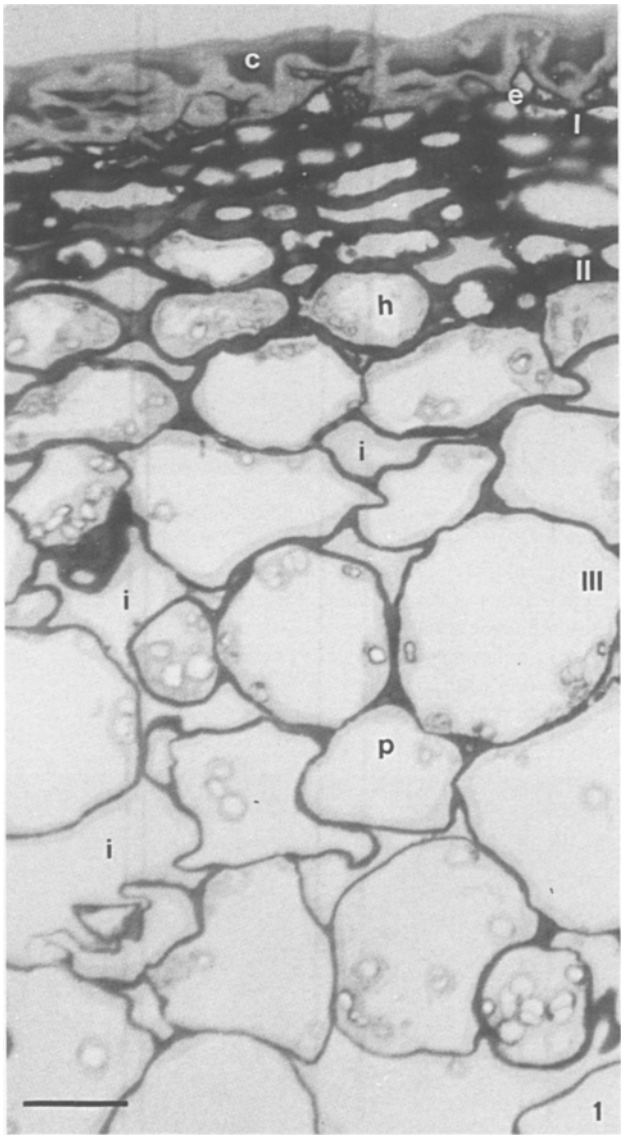

Fig. 1. Light micrograph of apple pericarp, which has been stained with toluidine blue, correlates calcium distribution and tissue morphology. Cellular zones of the apple pericarp include I epidermis (*e*) with cuticle (*c*), II hypodermis (*h*), and III parenchyma (*p*) where cell separation forms intercellular spaces (*i*). Bar: 25 μm

reached by the perfused fast green solution. As a result, we assumed that the ^{44}Ca solution also penetrated the three zones. Our SIMS observations were restricted to these three zones.

Ion microscopic images

The secondary ion images provided a map of the distribution of calcium across the section of the fruit. Sections of the water-treated fruits (Fig. 2) and of the ^{40}calcium-infiltrated fruit (Fig. 3) were analyzed.

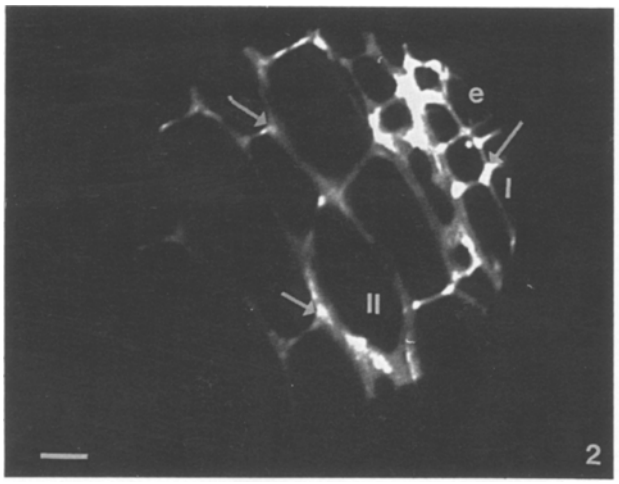

Fig. 2. Ion microscopic image revealing the distribution of naturally occurring calcium (^{40}Ca) in the pericarp of water-treated apples. Zones I and II. The calcium image shows a continuous signal outlining the contour of the cells. Note the strong signal where the middle lamella forms tricellular junctions (arrows). Tissue was chemically fixed by conventional procedures. Image integration time on the CCD camera was 120 s. *e* Epidermis. Bar: 25 μm

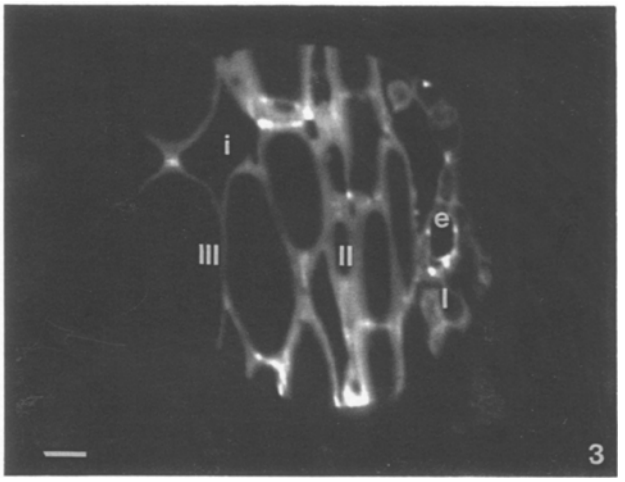

Fig. 3. Ion microscopic image of calcium ^{40}Ca in the pericarp of apples infiltrated with $^{40}CaCl_2$. A high calcium signal is obtained throughout the three zones (I, II and III) of the pericarp. Image integration time was 120 s. *i* Intercellular space, *e* epidermis. Bar: 25 μm

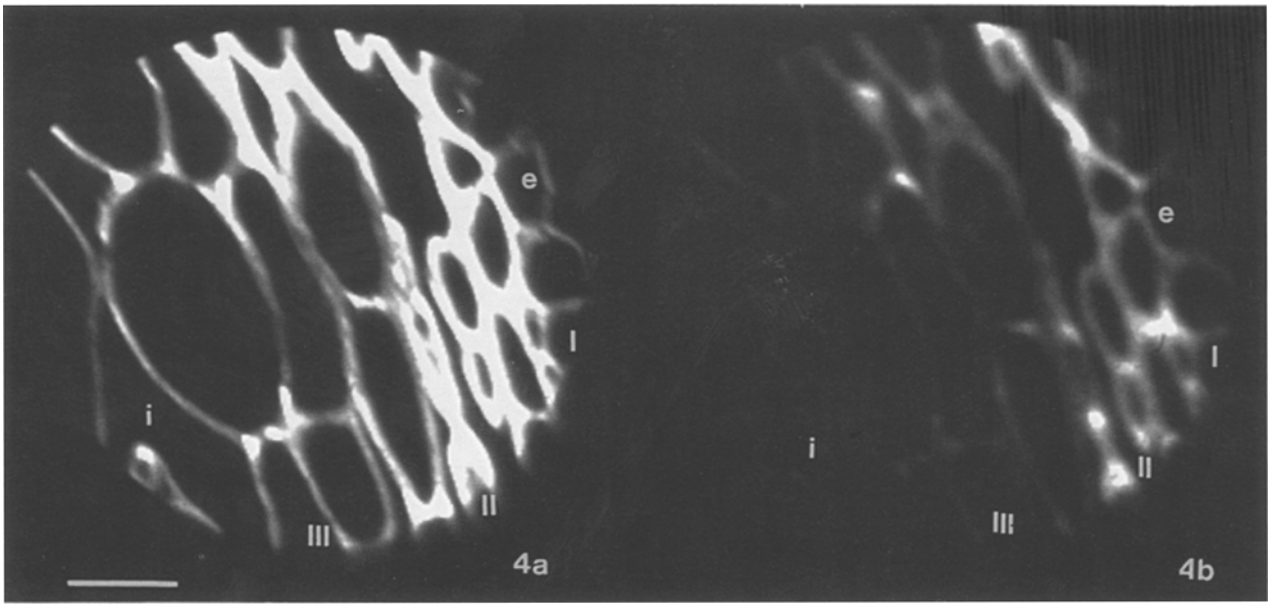

Fig. 4. a, b. Secondary ion image representing calcium distribution in the pericarp of apples infiltrated with $^{44}CaCl_2$. **a** Secondary ion image of ^{40}Ca (native calcium). **b** Secondary ion image of ^{44}Ca (exogenously applied calcium). The distribution of the secondary ion signal is different for the isotopes ^{40}Ca and ^{44}Ca. The exogenously applied calcium (mass 44) is mainly concentrated in zones I and II. Image integration time was 120 s. *i* Intercellular space, *e* epidermis. Bar: 25 μm

Brightness of the signal indicated relative ion intensity within a section. No significant differences in the signal intensity was obtained from the conventionally fixed and the freeze-fixed, freeze-substituted samples; however, the latter were more difficult to infiltrate with resin and to section. Therefore, all images illustrated in the results were obtained with the conventionally prepared samples.

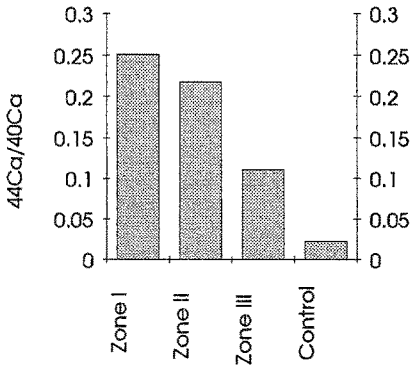

Fig. 5. Isotope ratio ($^{44}Ca/^{40}Ca$) calculated for zone I, the zone II, and the zone III of a section of the pericarp of apples infiltrated with $^{44}CaCl_2$ and in a non-infiltrated fruit (control)

The calcium signal clearly outlines the contours or walls of the cells but is rarely generated within the protoplasts. The apparent low signal intensity that is associated with the calcium in the cytoplasm may result from the high intensity generated by the cell walls and the limited dynamic range that can be represented in photographic reproductions. Although distinct bright spots were occasionally visible in a cell wall, no localized or concentrated distribution is observed in any specific zone (I, II, or III) across the pericarp of water-treated fruit (Fig. 2). In zone II, where the cells tightly adhere, the most intense signal for calcium is associated with the middle lamella at tricellular wall junctions (Fig. 2). The relative differences in the secondary ion signal intensity, i.e., counts per pixel, between the water-treated fruit (Fig. 2) and

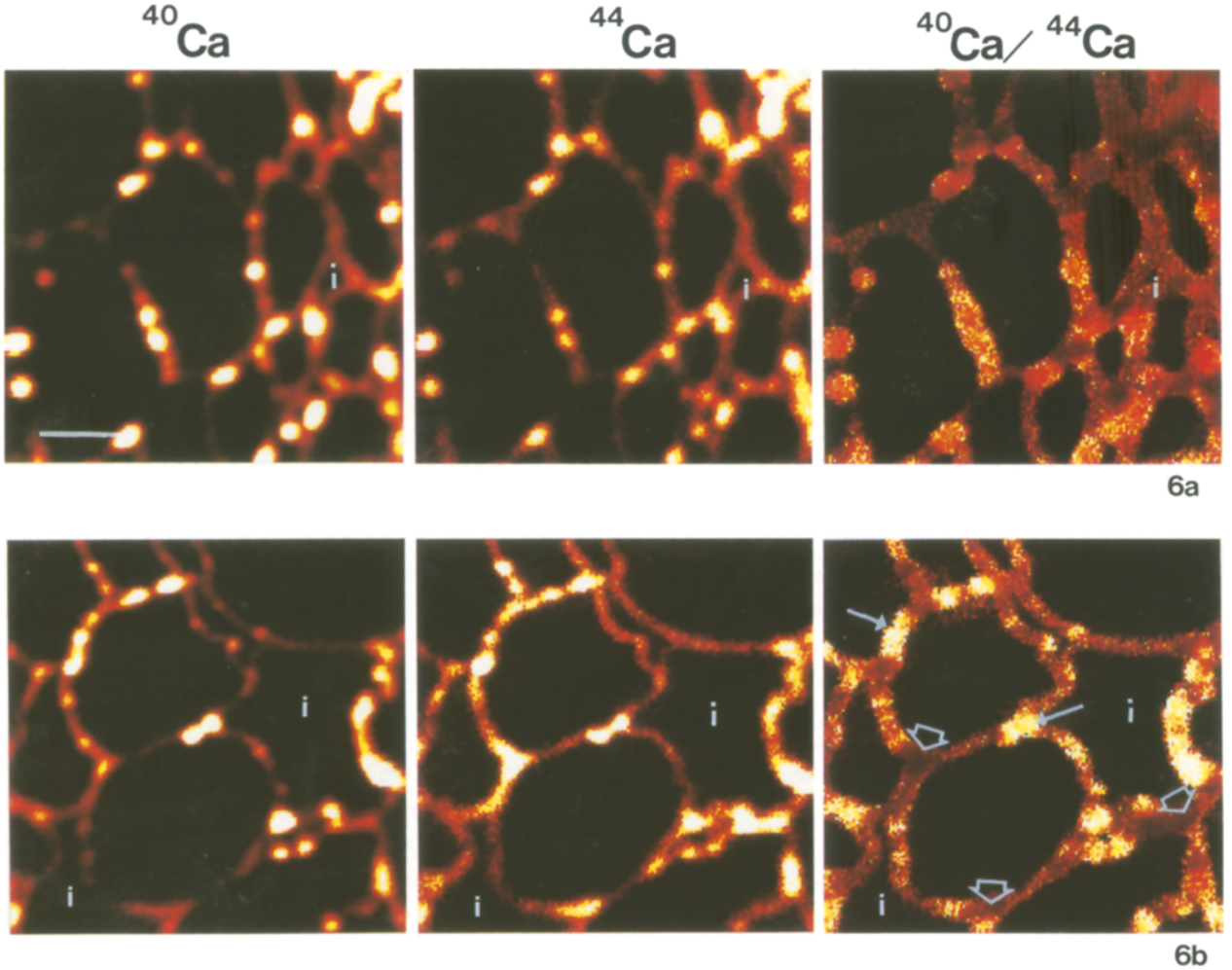

Fig. 6. a, b. Microprobe images of ^{40}Ca, ^{44}Ca, and the isotope ratio ($^{40}Ca/^{44}Ca$) of a section of the pericarp of apples infiltrated with $^{44}CaCl_2$. Images were produced by assigning a color from a continuous scale to each intensity of the $^{40}Ca/^{44}Ca$ ratio. Increase in ratio intensity is indicated by a color change from red to white. **a** Hypodermis (zone II of Fig. 1). ^{40}Ca and ^{44}Ca have the same distribution pattern. **b** Parenchyma (zone III of Fig. 1). Two distinct isotopic compositions can be distinguished around the same intercellular space (*i*): high $^{40}Ca/^{44}Ca$ (arrows) and low $^{40}Ca/^{44}Ca$ (arrowheads). Bar: 25 μm

the fruit infiltrated with ^{40}calcium (Fig. 3) indicate that ^{40}calcium accumulated within the cell wall after infiltration.

Ion microscope images of the pericarp of fruits infiltrated with ^{44}calcium are depicted in Fig. 4, which illustrates the location of naturally occurring calcium (mass 40; Fig. 4 a) and the exogenously applied calcium (mass 44; Fig. 4 b). Signal brightness represents the relative concentration of the isotopes in the sample. The results show that native calcium (^{40}Ca) is evenly distributed throughout the pericarp. Conversely, the exogenously applied calcium (mass 44) is mainly concentrated in zones I and II of the pericarp and only weakly found within the parenchyma (zone III). The localization of ^{40}Ca and ^{44}Ca within the cell walls is similar; both isotopes are associated with a high secondary ion signal that principally emanates from the middle lamella associated with tricellular junctions. In the control sample, which consists of the non infiltrated fruit, the same conditions and acquisition times provide an image of native ^{44}Ca that is barely visible.

Microprobe analysis and isotope ratio imaging

The absolute concentrations of calcium cannot be measured due to uncontrollable losses and possible redistribution of ions during fixation, embedding and sectioning. However, isotopic ratios can be calculated within a section by determining the measurements from random areas throughout the pericarp (Fig. 5). Calculating the ratios minimizes variations due to differential eroding of matrices. The ratio that was calculated for naturally occurring ^{44}Ca, which was obtained from a non-treated apple, was 0.022 ± 0.0001; this value is consistent with that found in the literature (Burns 1984, Chandra et al. 1990, Lazof et al. 1992). Ratios of $^{44}Ca/^{40}Ca$ in excess of the natural ratio of 0.022 indicate^{44}Ca incorporation. The samples of pericarp infiltrated with ^{44}Ca exhibit $^{44}Ca/^{40}Ca$ ratios that are significantly higher than the natural ratio; the outer layers of the pericarp (zone I and zone II from Fig. 1) have isotopic ratios ($^{44}Ca/^{40}Ca$) of 0.251 ± 0.00025 and 0.219 ± 0.00024, respectively, the value for zone III is 0.111 ± 0.00024 (Fig. 5). These isotopic ratios are consistent with the images from the ion microscope.

Figure 6 shows ^{44}Ca and ^{40}Ca images that were obtained from the pericarp using the microprobe mode of the SIMS and then integrated and divided ($^{40}Ca/^{44}Ca$) to obtain the isotope ratio image. The field of view for these images was 250 μm. The isotope images shown in Fig. 6 illustrate the spatially resolved information and quantitative relative isotopic intensity data. In zone II, the microprobe images of ^{40}Ca, ^{44}Ca, and $^{44}Ca/^{40}Ca$ not only confirm the uptake of ^{44}Ca by the cell walls, but also shows the same pattern of distribution between the native ^{40}Ca and the exogenously applied ^{44}Ca (Fig. 6 a). In zone III, where cells are separated by large intercellular spaces, the intensity relationship between the ^{44}Ca and ^{40}Ca indicates the occurrence of two distinct isotopic compositions in the cell wall (Fig. 6 b). Some of the high $^{40}Ca/^{44}Ca$ signal areas, corresponding to the points where the middle lamella splits to form intercellular spaces, have high native calcium content and low ^{44}Ca uptake (Fig. 6 b). Conversely, other wall areas along the same intercellular space have a very low ratio $^{40}Ca/^{44}Ca$ (Fig. 6 b). This observation suggests high ^{44}Ca uptake.

Discussion

During fruit ripening, cell wall polymer degradation is related to the enzymatic breakdown of pectins (Huber 1983, Brady 1987, Fischer and Bennett 1991). Pectins are complex carbohydrates that are involved in the evolution of firmness and cohesiveness of the tissues of fruits (Ferguson 1984, Van Buren 1991). The polyelectrolyte behavior of pectins has been well studied and the general idea that junction zones between pectic chains resemble fibrous solids is now well established in the literature (Jarvis 1992). Deesterification of the carboxyl groups of pectic galacturonic acid residues permits the formation of a calcium cross-linked network that envelopes the protoplast. This "egg-box" model for the junction zones of calcium pectate gels is compatible with the idea that this compound is rigid and insoluble.

Evidence of calcium uptake by the cell wall

The unique capability of SIMS to distinguish different isotopes of a chemical element allowed us to localize native calcium (mass 40) as well as exogenously applied calcium (mass 44) on the same section of embedded material. The distribution of native calcium was similar to that obtained with cherry tomatoes (Roy et al. 1994a) and flax epicotyl (Jauneau et al. 1992a), i.e., a high calcium signal emanated from the tricellular wall junctions. The use of stable ^{44}Ca, exogenously applied, provided a novel approach

for studying the uptake and distribution of applied calcium by the fruit.

During the last few years, the stable isotope ^{44}Ca has been used as a marker in SIMS studies that involve intestinal calcium absorption (Chandra et al. 1990), incorporation of calcium into bone (Bushinsky et al. 1990) or dentine (Lundgren et al. 1994), calcium exchange in cultured cells (Chandra et al. 1992, 1994) and quantification of nutrient tracers in plant tissues (Lazof et al. 1992). The low natural occurrence of ^{44}Ca found in our non-treated samples indicates that no interferences resulting from the combination of atoms present in tissues and having a mass very close to that of the labeling isotope interfere with our results and that ^{44}Ca was a suitable isotope to be used with fruit material.

The ^{44}Ca ion imaging provides evidence for the uptake and binding of exogenously applied calcium by the fruit. We believe that residual, unbound calcium, which would have been leached out during preparation and embedding, would not account for our results. This observation is supported by results from Jauneau et al. (1992a) whose pretreatment of samples with a calcium chelator totally eliminates the calcium signal in the plant tissue. This supports our contention that the ^{44}Ca signal represents the exogenously applied calcium that is bound to the cell wall.

The isotope ratio data illustrate the heterogeneity of domains that exist in the pericarp. The outer cortex of the fruit may contain as little as one-fifth of the calcium that is found in the peel and one-tenth of that present in the core (Ferguson 1984). Our data did not visualize significant ^{44}Ca in the inner layers of ther pericarp (0.111 $^{44}Ca/^{40}Ca$ ratio, vs. 0.251 for the external layers) because of this radial distribution. Bagshaw and Cleland (1993) have found a similar distribution in sunflower plants and a shift in calcium storage from the outer tissues to the inner tissues only in the presence of high levels of calcium. Apparently the cell wall forms calcium cross-bridges with relative ease in low calcium concentration areas.

Enriched areas of calcium, which occur beneath the epidermis, may have significance in retarding fruit decay. Many pathogens secrete pectolytic enzymes that attack the outer layers of the pericarp during the first phase of host–pathogen interactions; the infection thread is frequently initiated at a three-way cellular junction, i.e., a calcium rich spot. Perring and Wilkinson (1965) and Gormley (1981) have found that apples contain less calcium than would be required to saturate pectin in their cell walls. This observation would imply that the pectic junction zones in apple cell walls are single dimers (Jarvis 1984). Calcium pectate molecules, which exist initially as dimers, can be cross-linked by excess calcium ions (Powell et al. 1982, Thibault and Rinaudo 1986). Reinforcement of the architecture of the wall by applying exogenously calcium may help to create a natural barrier to infection.

Evidence for the involvement of calcium as a regulator of cell wall autolysis

The isotope ratio images, which were obtained with the microprobe analysis mode of the SIMS, suggests two types of microdomains in the cell wall. These microdomains, which occur at the intersects of the separating middle lamella where the intercellular spaces are formed, react differently in the presence of exogenously applied calcium. High $^{40}Ca/^{44}Ca$ ratio domains, i.e., low ^{44}Ca uptake, may occur when the Ca-pectin interaction has already formed. As a result, this area of the wall has strengthened making the intercellular spaces more resistant to further expansion. Conversely, areas having less Ca–pectin interaction may be able to capture and bind more ^{44}Ca. These domains may represent areas that were susceptible to wall separation, i.e., formation and expansion of intercellular spaces.

Whether or not the exogenously applied calcium can be exchanged for endogenous calcium or form new insoluble precipitates in the cell wall is a possibility that cannot be resolved by SIMS technology. However, in a recent study using affinity methods, exogenously applied calcium caused formation of new cross-bridges between pectic acids (Roy et al. 1994b). This process modified the distribution of anionic sites in the area where the middle lamella splits to form an intercellular space. Therefore, infiltrated ^{44}Ca does appear to be taken up and bound to the cell wall rather than exchanged.

Calcium ions that are bound to components of the cell wall, are not easily mobilized during sample preparation. Campbell et al. (1979) have found that nearly all of the cell wall calcium remained after fixation and dehydration. Wick and Hepler (1982) and Mentré and Escaig (1988) confirmed that calcium remained in situ during aldehyde fixation. Touchard et al. (1987) demonstrated very low mobility of calcium in the cell wall. Jauneau et al. (1992b) suggested that the degree of calcium mobility in the cell wall appeared to relate

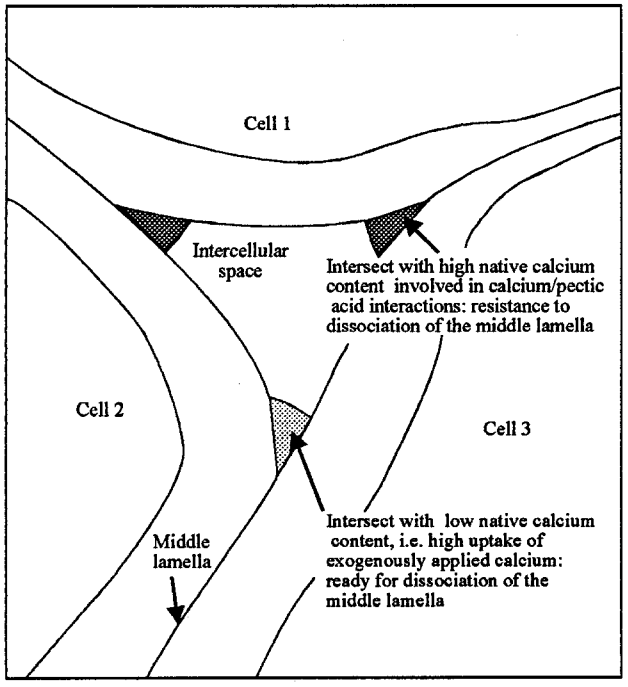

Fig. 7. Diagram of an intercellular space between three adjacent cells in the fruit pericarp. The model suggests that cell separation, which forms intercellular spaces, results from the dissolution of the middle lamella. The intersects may react differently during the expansion of one intercellular space, because of the availability of calcium ions

to the degree of esterification of the pectin. In earlier studies, we also concluded that calcium distribution in fruit tissues, where pectins are mainly acidic, did not seem to be affected by the sample preparation (Roy et al. 1994a). Because our study deals with calcium distribution following infiltration with calcium, redistribution of loosely bound and free infiltrated calcium was considered. We followed the suggestions of Burns (1982), Chandra and Morrison (1992), and Lazof et al. (1994) who recommended that freeze-fixation has to be used for samples that are analysed by SIMS. In our studies samples were freeze-fixed and freeze-substituted. This procedure appeared to preserve the morphological and chemical integrity of the specimen.

Figure 7, which summarizes our results, suggests the existence of microdomains in the cell wall around the same intercellular space. At some middle lamella intersects, calcium ions may stabilize junctures between adjacent cells, thereby limiting or preventing cell separation. However, at other middle lamella intersects, the low concentration of calcium would facilitate the formation and expansion of the intercellular spaces. These results suggest a possible role of target areas in the intercellular space involved in the control of cell separation.

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